Microbiology, Vol. 70, No. 1, 2001, pp. 36–40. Translated from Mikrobiologiya, Vol. 70, No. 1, 2001, pp. 45–50.

EXPERIMENTAL ARTICLES

Role of the Polysaccharide Components of *Azospirillum brasilense* Capsules in Bacterial Adsorption on Wheat Seedling Roots

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Received February 28, 2000; in final form, April 3, 2000

Abstract—*Azospirillum brasilense* cells deprived of capsular exopolysaccharides completely lost their ability to bind wheat germ agglutinin (WGA) and much of their ability to attach to wheat seedling roots. The decapsulation of bacterial cells by washing them with a NaCl solution led to an increase in the relative hydrophobicity of the cell surface. The pretreatment of wheat seedling roots with *N*-acetyl-D-glucosamine (GlcNAc) or the GlcNAc-containing polysaccharide complexes stripped from *Azospirillum* cells reduced their attachment to the roots. Under the experimental conditions used (3-h incubation of wheat seedling roots with exponential-phase azospirilla), bacterial adsorption is mainly driven by the specific mechanisms attachment of the cells to the roots, whose operation is due to the capsular polysaccharide components and the WGA present on the wheat seedling roots.

Key words: *Azospirillum brasilense*, capsule, extracellular polysaccharide, adsorption, wheat.

Bacteria of the genus *Azospirillum* are extensively studied in relation to their ability to beneficially influence the growth and productivity of crops. It was found that the bacterial cell surface plays an important role in the contact interaction of these bacteria with the host plant roots at its early stages. The capsular material of azospirilla contains carbohydrates with a high affinity for calcofluor [1], Congo Red [2, 3], and FITC-labeled lectins [4], as well as lipopolysaccharides (LPSs) [5], agglutinins [6], proteins responsible for the hydrophobicity of the cell surface [7], and complex polysaccharides that can be released into the surrounding medium [3]. The complex age-dependent [8, 9] composition of the capsular material enables the bacterial capsule to perform multiple functions, which are efficiently studied using mutants deficient in the synthesis of the particular capsular component. However, the study of such mutants sometimes gives results that are difficult to interpret unambiguously. For instance, the absence of polar flagellum results in a decrease in the ability of azospirilla to colonize roots [10]. However, it remains unclear whether this is due to defects in the structures responsible for the binding of azospirilla to roots or to the loss of bacterial motility.

In the present study, the role of the capsular material, as well as its particular high-molecular-weight polysaccharides, of *A. brasilense* cells in their attachment to wheat seedling roots was studied using an alternative approach: comparison of the strength of the attachment of encapsulated and non-encapsulated cells.

MATERIALS AND METHODS

The strain *Azospirillum brasilense* Sp245 was a generous gift from J. Dobereiner (Brazilia) [11]. The strain *A. brasilense* 80, isolated from the wheat cultivar Saratovskaya 49 roots [12], was obtained from the Collection of the Institute of Biochemistry and Physiology of Plants and Microorganisms, Saratov.

To prepare surface polysaccharides, *A. brasilense* cells were grown to the late exponential phase in a malate-containing synthetic medium in an Ankum-2M fermentor as described previously [3]. Polysaccharides were extracted from the surface of the cells by incubating them at 4°C for 72 h in 0.15 M NaCl containing 0.02% sodium azide with continuous stirring. At 24-h intervals, the cells were harvested by centrifugation and transferred to a fresh saline solution. The first three polysaccharide extracts were pooled and dialyzed in a cellulose sack with a cut-off level of 12–14 kDa (Sigma, United States). The dialyzed extract was then fractionated on a column (55×1.8 cm) packed with Sepharose CL-4B (Pharmacia, Sweden) using 0.025 M NH_4HCO_3 (pH 8.1) as the eluant. Carbohydrates were detected by the reaction with phenol and sulfuric acid and proteins were detected by the absorbance measured at 280 nm on an SF-46 spectrophotometer (LOMO, Russia). The different types of the polymers obtained were arbitrarily designated as lipopolysaccharide–protein complexes (LPPC) and polysaccharide–lipid complexes (PSLC) [3]. The complexes were analyzed for the content of carbohydrates, proteins, nucleic acids,

0026-2617/01/7001-0036$25.00 © 2001 MAIK "Nauka / Interperiodica"

and 2-keto-3-deoxyoctonate (KDO), according to procedures described elsewhere [3].

Bacterial adsorption experiments were carried out using freshly grown encapsulated cells suspended in a buffer solution and cells deprived of capsules by incubating them, with continuous stirring, for 7 days in phosphate-buffered saline (PBS) of the following composition (g/l): KH_2PO_4, 0.43, Na_2HPO_4, 1.68; and NaCl, 7.2 (pH 7.2). At 24-h intervals, the cells were harvested by centrifugation and transferred to fresh PBS. After cell precipitation, each of the supernatants was concentrated ten times by evaporating it under a vacuum and analyzed by the double-immunodiffusion method with antibodies against glutaraldehyde-treated azospirilla [5]. Suspensions of encapsulated and non-encapsulated cells were equalized in cell density, which was estimated from the optical density of suspensions measured at 420 nm in 1-cm-pathlength cuvettes on a Specol photoelectric colorimeter (Karl Zeiss, Germany). To determine the number of viable cells, 0.1-ml aliquots of bacterial suspensions with optical densities of 1.4 were serially diluted in PBS, and the last dilution was plated, in triplicate 0.1-ml amounts, onto malate-containing agar. The colonies were counted after incubating the plates at 30°C for 48 h.

Adsorption experiments were carried out using the soft spring wheat *Triticum aestivum* cultivars Saratovskaya 58 and 29, which are maintained at the Research Agricultural Institute of Southeastern Russia in Saratov. Wheat seeds were thoroughly washed with water and sterilized in 70% ethanol for 1 min and in 5% sodium hypochlorite for 5 min. The seeds were placed in petri dishes and incubated in the dark at 25°C for 3 days to induce germination. Then, 2-cm-long pieces of roots cut from their apical ends were aseptically transferred to tubes (one piece per tube) containing 4.5 ml of PBS and inoculated with 0.5 ml of the respective bacterial suspension containing about 10^8 cells/ml. After incubation on a shaker at 30°C over different time periods, the root pieces with adsorbed azospirilla were washed three times with PBS while gently shaking them and then homogenized. The homogenate was serially diluted and the dilutions were plated onto malate-containing agar to determine the number of grown colonies and, hence, the number of azospirilla adsorbed on the root surface [3].

To evaluate the effect of GlcNAc on the adsorption of *Azospirillum* cells to wheat roots, the latter were incubated in 15 and 30 mM solutions of the amino sugar for 1 h. The root pieces were then incubated with azospirilla over different time periods (from 0.25 to 24 h) and assayed for the number of adsorbed bacterial cells as described above. The effect of capsular polysaccharide complexes on bacterial adsorption was studied in a similar way. Namely, root pieces were preincubated in a solution containing a particular complex at a concentration of 1 mg/ml (for PSLC, this weight concentration corresponded to a molar concentration of 2 μM). The preincubated roots were then incubated with azospirilla cells for 3 h. Aliquots for enumerating the bacteria were taken at the beginning and at the end of incubation.

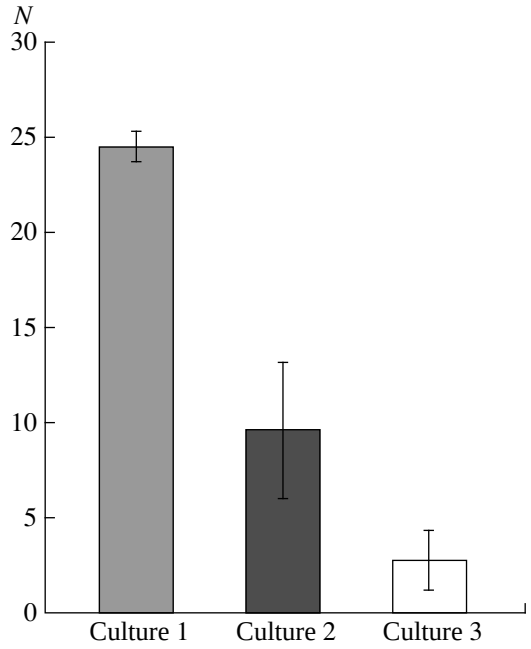

Fig. 1. Adsorption of encapsulated (culture 1 and culture 2) and decapsulated *A. brasilense* Sp245 cells on the wheat cultivar Saratovskaya 29 seedling roots. *N* represents the number of azospirilla ($\times 10^5$) adsorbed on 1 cm of root.

The results of the experiments were statistically processed and confidence limits were determined for a confidence level of 95%.

The hydrophobicity of encapsulated and non-encapsulated azospirilla was determined by two methods [13]. Hydrophobic chromatography on Phenyl-Sepharose yielded the hydrophobicity index, which represents the number of adsorbed cells on the hydrophobic matrix expressed as a percentage of the total number of cells. A salting-out test yielded the minimal concentration of ammonium sulfate (in %) that causes cell aggregation.

Transmission electron microscopy was carried out using a Tesla BS-500 electron microscope at an accelerating voltage of 70 kV. Encapsulated and non-encapsulated cells were mounted on a Formvar-coated copper or nickel grid and incubated for 20 min in a drop of phosphate buffer containing 0.2% bovine serum albumin and 0.02% Tween-20. Then, the grids were washed with the same buffer from which the albumin was omitted, incubated for 30 min in a solution of colloidal gold–labeled wheat germ agglutinin, washed with the buffer and then with bidistilled water, and examined in the electron microscope.

RESULTS AND DISCUSSION

The role of capsular material in the adsorption of azospirilla to wheat roots was studied using *A. brasilense* Sp245 cells grown in a malate-containing liquid

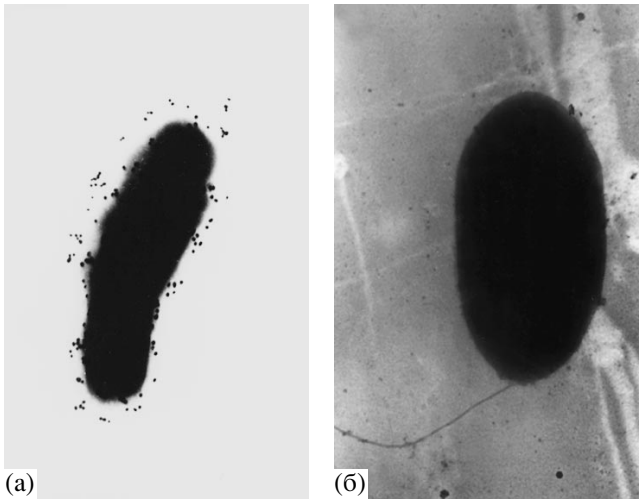

Fig. 2. Transmission electron microscopy of (a) encapsulated and (b) decapsulated *A. brasilense* Sp245 cells treated with colloidal gold–labeled WGA (10000×).

medium to the late exponential phase (20 h of growth). Bacterial cells were deprived of capsules by incubating them in PBS for 7 days, as described in the *Materials and Methods* section.

In these experiments, the completeness of capsule removal from the bacterial cells was of crucial importance, and was controlled by analyzing the PBS used for the capsule removal every 24 h by the double-immunodiffusion method [5]. Analysis showed that, as soon as after 6–7 days of incubation, PBS contained no capsule polysaccharides, even if concentrated solutions were used for the analysis. In this case, decapsulated cells remained motile and could partially restore their capsules after 3–4 days of incubation at rest in the buffer.

In preliminary experiments, the dynamics of cell attachment to wheat roots was studied. As soon as after 15-min incubation of the roots in a bacterial suspension containing 6.5×10^8 cells/ml, the amount of adsorbed cells reached $(3.7 \pm 1.2) \times 10^5$ cells/cm of root. After 3 h of incubation, the adsorption was maximum and amounted to $(2.6 \pm 0.5) \times 10^6$ cells/cm of root, which virtually did not change over the course of the further incubation. Based on these results, further adsorption experiments were carried out using the 3-h incubation of the roots with three types of cells: fresh 20-h encapsulated cells, encapsulated cells stored in PBS at 4°C for 7 days, and decapsulated cells (in Fig. 1, the suspensions of these cells are designated as culture 1, culture 2, and culture 3, respectively). As a result of long-term storage without changing the buffer, culture 2 presumably contained metabolically inactive cells. As can be seen from the histogram presented in Fig. 1, the degree of adsorption of fresh encapsulated cells and encapsulated cells stored in PBS for 7 days was, respectively, 9 and 3.5 times higher than that of decapsulated cells.

Table 1. Relative hydrophobicity of encapsulated and decapsulated *A. brasilense* Sp245 cells

Cells	Hydrophobicity index*, %	Salting-out test**, %
Encapsulated	49.7 ± 3.3	42
Decapsulated	68.9 ± 2.8	36

*The hydrophobicity index was determined by hydrophobic chromatography as described in the *Materials and Methods* section.

**The results of the salting-out test was expressed as the minimal concentration of ammonium sulfate (in %) that caused cell aggregation.

It is known that bacterial adsorption is due to both nonspecific interactions related to the charge and hydrophobicity of contact surfaces and to the specific interactions of surface biomolecules.

The hydrophobicity data presented in Table 1 show that the decapsulation of cells led to an insignificant increase in the relative hydrophobicity of the cell surface. This observation somewhat disagrees with the data of Castellanos *et al.* [7] and Dufrene and Rouxhet [9], who found that the increased hydrophobicity of the cell surface, as a rule, promotes bacterial adhesion.

The root surface is typically covered with a layer of negatively charged acidic polysaccharides. *Azospirillum* cells are also negatively charged [7]. One might assume that the attraction of the likely charged surfaces of roots and bacterial cells is mediated by divalent cations. However, as shown by Gafny *et al.* [14], the presence of mono-, di-, and polycations inhibits the adsorption of azospirilla. It is possible that some specific molecules of bacterial capsules can aid in overwhelming the repulsion of the likely charged surfaces. To verify this supposition, we examined the interaction of azospirilla with the colloidal gold-labeled wheat germ agglutinin (WGA) by transmission electron microscopy. The photomicrographs presented in Fig. 2 show that, unlike encapsulated *A. brasilense* Sp245 cells, decapsulated cells were unable to interact with WGA. These data suggest that it is the removal of some surface substances capable of interacting with WGA that may be responsible for the decrease in the adhesiveness of azospirilla. The location of binding sites for wheat germ agglutinin in the capsular material of azospirilla was also reported by Del Gallo *et al.* [1].

The role of WGA–carbohydrate interactions in the attachment of azospirilla to wheat seedling roots was investigated in the experiments with *A. brasilense* 80 cells and the wheat cultivar Saratovskaya 58 seedling roots. It was found that GlcNAc (a specific WGA hapten) inhibits bacterial adsorption on the wheat roots: a 2-h incubation period of the roots with 15 and 30 mM GlcNAc inhibited the adsorption by 1.4 and 7.5 times, respectively. The inhibitory effect of GlcNAc was absent when the incubation time was less than 1 h or

Table 2. The inhibitory effect of GlcNAc and capsular polysaccharide complexes on the attachment of *A. brasilense* cells to wheat seedling roots

A. brasilense strain	Preparation	Number of cells per cm of root
80	Control	$(2.06 \pm 0.58) \times 10^5$ (15)
	30 mM GlcNAc	$(2.27 \pm 0.93) \times 10^4$ (15)
80	Control	$(1.94 \pm 0.84) \times 10^5$ (24)
	LPPC	$(4.98 \pm 2.58) \times 10^4$ (20)
	PSLC	$(3.20 \pm 0.79) \times 10^4$ (24)
Sp245	Control	$(2.63 \pm 0.47) \times 10^6$ (37)
	LPPC	$(2.41 \pm 0.94) \times 10^5$ (33)
	PSLC	$(2.78 \pm 1.45) \times 10^5$ (27)

greater than 12 h, indicating that the specific WGA-dependent mechanism of bacterial adsorption on roots is active within these limits of incubation time. The inhibitory effect of GlcNAc was maximum when this amino sugar was incubated with the roots and cells for 3 h. The data presented in Table 2 show the important role of the cell surface components in the adsorption of azospirilla on wheat roots. The significance of microbial metabolism in bacterial adsorption was shown by Bashan and Holguin [15]. Depending on the time and conditions of incubation, different adsorption mechanisms can be involved. For instance, Gafny *et al.* [14] showed that not only GlcNAc but also amino acids (lysine, alanine, and glutamic acid) and some sugars can inhibit the adsorption of azospirilla.

The nature of the WGA receptors present on the surface of azospirilla remains unknown, although it would be reasonable to suggest that these are carbohydrate-containing substances. We isolated and attempted to evaluate the role in adsorption of particular surface high-molecular-weight polysaccharides, the so-called LPPC and PSLC, which are localized in the capsules of azospirilla and may be released into the surrounding medium. The chemical compositions of these surface complexes are shown in Table 3. LPPC and PSLC were separated by gel filtration on a Sepharose CL-4B column. LPPC was eluted in the void volume of the column, indicating that the molecular mass of this complex is at least several thousand kDa. At the same time, the molecular mass of PSLC corresponded to a 500-kDa dextran. LPPC contained a smaller amount of carbohydrates and a greater amount of KDO, which is a specific component of lipopolysaccharides, than PSLC. Thin-layer chromatography showed that the carbohydrate moieties of the LPPC and PSLC of strain Sp245 contain rhamnose, galactose, galacturonic acid, xylose, glucosamine, and galactosamine. The complexes of strain 80 contained rhamnose, galactose, galacturonic acid, and glucosamine. Taking into account that GlcNAc inhibits bacterial adsorption and that both the LPPC and PSLC complexes of either *A. brasilense* strain contain glucosamine, which is responsible for the interaction of azospirilla with WGA [3, 16], we investigated the effect of LPPC and PSLC on adsorption.

Experiments were carried out with two contact pairs: (a) *A. brasilense* 80 cells and wheat cultivar Saratovskaya 58 seedling roots and (b) *A. brasilense* Sp245 cells and wheat cultivar Saratovskaya 29 seedling roots (Table 2). The pretreatment of the roots with the LPPC and PSLC of strain 80 inhibited bacterial adsorption by 3.9 and 5.9 times, respectively, while the LPPC and PSLC of strain Sp245 inhibited adsorption by 10.9 and 9.4 times, respectively. Control experiments showed that neither glucosamine nor the polysaccharide complexes influenced the reproduction of bacteria in the incubation medium.

As for the mechanism of action of LPPC and PSLC on bacterial adsorption, it is possible that these complexes inhibit adsorption by blocking the specific binding sites on the wheat roots due to lectin–carbohydrate interactions. The detection of KDO in LPPC indicates that this surface complex contains a lipopolysaccharide. The lipopolysaccharide of the cell membrane is not involved in the specific binding of WGA, as judged from the results of experiments with decapsulated *Azospirillum* cells. It remains unclear whether the lipopolysaccharide of LPPC acquires the ability to bind to WGA when it appears in the capsular material (such possibility was shown for some gram-negative bacteria [17]), or the lipopolysaccharides of LPPC and cell membrane differ in their properties. The investigation of this problem is in progress in our laboratory.

Thus, the results obtained in this study indicate that the WGA present on the wheat seedling roots, as well as the LPPC and PSLC (or their components) of the

Table 3. Composition of the high-molecular-weight polysaccharide complexes isolated from capsules of azospirilla

A. brasilense strain	Complex	Content, wt %			
		carbohydrates	proteins	nucleic acids	KDO
Sp245	LPPC	24.8 ± 1.2	22.1 ± 0.2	0.5 ± 0.02	1.5 ± 0.10
	PSLC	57.0 ± 1.0	12.0 ± 0.1	1.7 ± 0.04	0.5 ± 0.02
80	LPPC	25.3 ± 1.2	18.8 ± 0.4	0.3 ± 0.03	3.0 ± 0.11
	PSLC	45.8 ± 1.1	15.9 ± 0.8	0.5 ± 0.04	0.6 ± 0.12

capsule material of azospirilla, are involved in cell attachment to the wheat roots.

ACKNOWLEDGMENTS

We are grateful to L.Yu. Matora and O.B. Serebrennikova for immunodiffusion analysis.

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